Time-Restricted Feeding Alters the Innate Immune Response to Bacterial Endotoxin

Undergraduate Thesis Research

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By

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Abstract

Circadian rhythms have a regulatory effect on immune function. The immune system has circadian fluctuations in gene expression regulated by circadian clock proteins activation of important transcription factors in the immune system. Experimentally induced circadian disruption, through both genetic ablation of clock proteins and exposure to light at night impairs immune function. Although light is the primary entraining factor for circadian rhythms, timed feeding can entrain peripheral clocks. Time restricted feeding can shift circadian rhythm function in the liver independent of light and the SCN. Time restricted feeding has been used to study the effects of circadian rhythms on metabolic diseases, but the relationship between time restricted feeding and immune function is unknown. Because of this, we tested the hypothesis that temporal desynchrony of food intake alters immune responses. Sixty adult male Swiss Webster mice were given food either at night, during the day, or *ad libitum*. After four weeks, half the mice received an injection of lipopolysaccharide (LPS) as an immune challenge and half received a saline injection. Three hours later liver, adrenal, and overall mass were all measured. Cytokine gene expression was measured by using qPCR in the liver, spleen, and hypothalamus. The bacteria killing capacity of the serum against *Escherichia coli* was measured as a functional immunity assay. Mice that only had access to food during the day, the 'wrong' feeding time for nocturnal animals, had suppressed cytokine response and did not have the enhanced bacteria capacity in response to LPS in comparison to night restricted or *ad-lib* fed animals. These data suggest that mistimed feeding has a detrimental effect on immune function independent of body mass, and provide further evidence for the integration of the circadian, metabolic, and immune functions.

Introduction

Light is the strongest entraining signal of circadian rhythms. Light is detected by intrinsically-photosensitive retinal ganglion cells (iPRGCs) in the retina and a signal is sent via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) of the hypothalamus. Organs have cell-autonomous oscillations driven by a transcriptional-translational feedback loop of clock genes (1,2). This feedback loop starts when two transcription factors, CLOCK and BMAL1 heterodimerize. This dimer then transports into the nucleus and binds to DNA, initiating transcription of genes containing E-box regulatory elements including Per and Cry genes. When translated, these proteins dimerize and form complexes with other proteins. This PER:CRY complex builds up and feeds back to inhibit the effect of the CLOCK:BMAL dimer on their own transcription. Over time the inhibitory complexes are broken down and gene transcription can take place again (3). Because peripheral organs cannot receive light information directly, the SCN sends signals through endocrine and sympathetic pathways which entrains their clocks to the time of day (4).

Circadian rhythms are prevalent in most organs and functions in the body, including the immune system. In mice, macrophages contain intrinsic circadian clocks that operate autonomously and control inflammatory immune response. Circadian genes such as Cry and Rev-erbα regulate the expression of proinflammatory cytokines including Ccl2, IL-6, TNF-α, and iNOS (5-9). Core clock genes also regulate immune system transcription factors such as the signal transducer and activator of transcription 3 and 5 (*stat3 & stat5*), early growth response gene 1 (*egr1*), and nuclear factor kappa B (NF-κB) (10,11). The SCN transmits timing information to the immune system through both endocrine and autonomic pathways (4). Circadian rhythm disruption through clock gene ablation, phase advances, or dim light at night

disrupts the inflammatory response including expression of cytokines by disrupting these communication pathways (12-15).

With the invention of artificial lighting, people are living in a 24/7 society. There are no longer restrictions in activity due to lack of light, and food is available at any given time, day or night. While light is the most important factor in entraining circadian rhythm, timing of food intake can also shift peripheral clocks. Restricting feeding to a specific time during the light cycle shifts circadian oscillations in the liver independent of SCN or light/dark cycle (16,17). Because circadian rhythm disruption maintains metabolic homeostasis, labs have used timed feeding to investigate the link between circadian rhythm and metabolic diseases such as diabetes and obesity independent of caloric intake (18,19). Environmental circadian rhythm disruption is known to affect immune function (20,21), and timed feeding can shift peripheral clocks, but the effects of time restricted feeding on the immune system is unknown.

To test the effect of time restricted feeding on immune function 60 adult male Swiss-Webster mice were given food either only during the night, during the day, or *ad-libitum* for four weeks. After the four weeks, half the mice were injected with one intraperitoneal (i.p.) dose (0.5 mg/kg) of lipopolysaccharide (LPS; AKA endotoxin), or saline at the onset of their active period. Spleen, liver, adrenals, and brain were collected three hours later. Overall mass, adrenal mass, and spleen mass were measured. PCR was used to examine the expression of cytokines in the liver, spleen, and hypothalamus. The hypothalamus was examined due to its importance in coordination of sickness behavior through cytokine expression in response to peripheral immune activation. A bacterial killing assay was performed as a functional test of immunity. The day-fed animals did not have the same increase in bacteria killing capacity in response to LPS as the

other feeding groups did. This was reflected by a lesser cytokine response to LPS in the spleen. Cytokines in the liver and the hypothalamus did not show the same dampened response.

Methods

Animals

60 adult male (>8 wks old) Swiss Webster (Charles River Laboratories, Kingston, NY) mice were used for all experiments; a separate set of 36 mice was utilized for the bacteria killing assay. Mice were individually housed and allowed to recover for 1 week after arriving to our facility to entrain to a 12:12 light/dark cycle (09:00-21:00 EST) and recover from the stress of shipping. The following week all mice were habituated to twice daily cage switching without food restriction in order to acclimate to this procedure. Mice were then randomly assigned to one of three groups: day-fed, night-fed, or *ad libitum*-fed (Ad lib). Day-fed mice were allowed access to standard rodent chow (Harlan, Teklad #7912) during the 12 h light phase, after which they were transferred to a new cage which contained only water and no food. After the 12 h restriction period, mice were placed back into their food-containing cage. This was done to prevent 'crumbling 'or 'hoarding' behavior which may allow animals to eat outside of the restricted time frame. Night-fed animals were similarly only allowed food during the 12 h dark phase, and transferred to their second cage with water immediately at the beginning of the light phase. Ad lib animals were allowed access to food at all times, but also experienced twice-daily cage changes to control for the stress of this manipulation.

Endotoxin Administration and Tissue Collection

After four weeks of timed food restriction, mice received an i.p. injection of 0.5 mg/kg LPS (serotype 0111:B4; Sigma Aldrich, St. Louis, MI) or sterile saline one hour prior to the onset of the dark phase (ZT11-12). Three hours later, mice were rapidly decapitated and blood was collected. Blood was centrifuged at 4 °C and serum was removed and stored at -80 °C until multiplex assay. Brains and spleens were also collected and stored for pPCR analysis.

Quantitative Polymerase Chain Reaction (qPCR)

Following euthanasia via cervical dislocation, brains were immediately dissected, cut in half (sagittally), and placed into RNALater reagent (Qiagen) on ice. Hypothalami were further dissected one week later for gene expression analysis. RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and quantity were determined using a spectrophotometer (NanoDrop), and cDNA was synthesized using M-MLV reverse transcription. 40 ng of cDNA/reaction was used in subsequent PCR. Taqman Fast advanced master mix (Life Technologies) containing AmpliTaq Fast DNA polymerase was used in a 20 uL duplex reaction with one of the primer/probe pairs listed in table 1 and a primer-limited primer/probe for the endogenous control eukaryotic 18s rRNA. The 2-step real-time PCR cycling conditions used were: 95°C for 20 s, 40 cycles of 95°C for 3 s, and then 60°C for 30 s. Relative gene expression was calculated using the Pfaffl method(19).

Bacteria Killing Assay (BKA)

The bacteria killing assay is an *ex vivo* assessment of innate immunity mediated by complement proteins. Samples were immediately centrifuged at 4°C for 25 min at 4000x*g* and serum aliquots were stored at −80 °C until assayed. Under a laminar flow hood, serum samples were diluted 1:20 in CO_2-independent media (Gibco, Carlsbad, CA, USA). A standard number of

colony-forming units (CFUs) of *Escherichia coli* (Epower 0483E7, Fisher Scientific) was added to each serum sample in a ratio of 1:10. Plasma-bacteria mixtures were then incubated for 30 min at 37°C, and plated in duplicate onto tryptic-soy agar plates (Teknova, Hollister, CA, USA) using sterile technique. Two plates were spread with diluted bacteria alone as positive controls, and two were spread with media alone as negative controls. All plates were incubated at 37°C overnight, and then total CFUs were quantified by an experimenter unaware of group assignments. Total CFUs were averaged across the duplicates for each animal and then compared with the average of the positive control plates to calculate the per cent of bacteria killed. Neither negative control plate contained CFUs.

Statistical Analyses

Differences in body mass were analyzed using a one-way ANOVA. Two way ANOVAs, assessing the effects of time of feeding, injection, and interactions, were conducted on somatic measures and bacteria killing capacity. If the data did not meet the assumptions of normality or equal variance (gene expression and protein concentrations), then nonparametric tests (Mann-Whitney and Kruskal-Wallis tests) were conducted, followed by a Dunn's post-hoc test. Samples were excluded from analysis if 18S CT value was greater than 18. Mean differences were considered statistically significant when ≤ 0.05 for all analyses. Statistical analyses were conducted using SPSS Statistics v 22 (IBM; Armonk, NY).

Results

Somatic Measures

After four weeks of timed food restriction mice exhibited no differences in body mass or adrenal mass ($p > 0.05$: Fig 1A and 1B, respectively). Spleen mass increased in response to LPS in all groups ($F_{1,53} = 8.48$; $p < 0.01$; Fig 1C). Body mass of both night and ad-lib fed mice decreased three-hours after LPS injection, suggesting anorexia in response to LPS ($F_{1,18}= 50.12$, $p < 0.001$ and $F_{1, 18}= 10.62$, $p < 0.05$, respectively). Day-fed animals did not exhibit this body mass decrease ($p > 0.05$; Fig 1D).

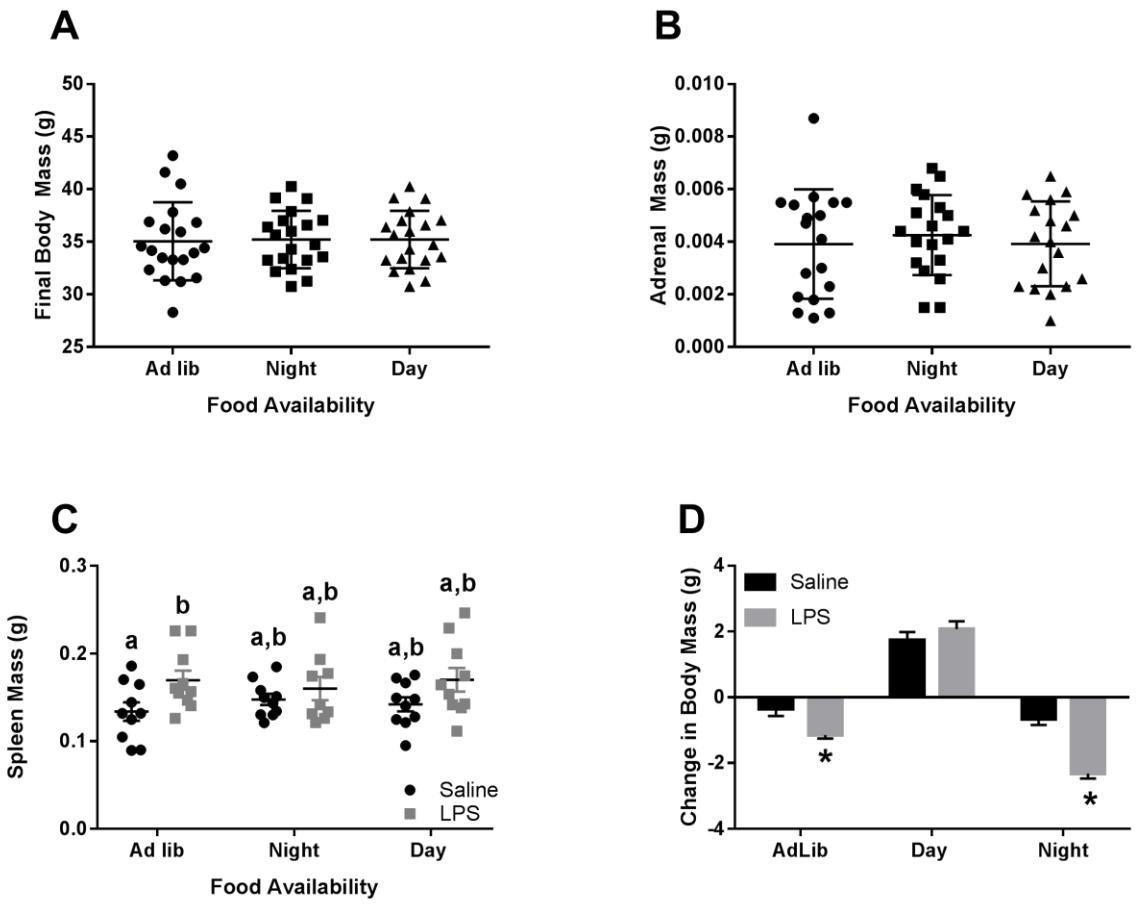

Figure 1

Spleen

LPS injection increased splenic expression of TNF-α, IL-1β, and IL-6, ($U = 41.00$, $p < 0.001$; $U = 91.00$, $p < 0.001$; $U = 35.00$, $p < 0.001$; Fig. 2A, B, and C respectively). Timed feeding did not alter splenic cytokine expression, but did interact with LPS ($X^2 = 28.03$, $p < 0.001$; $X^2 = 21.73$, $p < 0.001$; $X^2 = 31.45$, $p < 0.001$; respectively), such that day-fed mice decreased cytokine response to LPS relative to saline-injected mice (Dunn's test, $p > 0.05$). LPS increased expression of TNF- and IL-6 in ad lib mice (Dunn's, $p < 0.05$ and $p < 0.01$). LPS increased expression of TNF-α, IL-1β, and IL-6 in night-fed mice (Dunn's, $p < 0.05$ in all cases). Timed feeding did not affect expression of TNF-α, IL-1β, and IL-6 in the spleen of saline treated mice regardless of feeding group ($p > 0.05$).

Liver

LPS injection increased hepatic expression of TNF-α, IL-1β, and IL-6, ($U = 2.00$, $p < 0.001$; $U = 7.00$, $p < 0.001$; $U = 0.00$, $p < 0.001$; Fig. 2 D, E, and F respectively). Timed feeding did not alter hepatic cytokine expression, but did interact with LPS ($X^2 = 40.29$, $p < 0.001$; $X^2 = 38.35$, $p < 0.001$; $X^2 = 42.29$, $p < 0.001$; respectively). Timed feeding did not affect expression of TNF-α, IL-1β, and IL-6 in the livers of saline treated mice regardless of feeding group ($p > 0.05$).

Hypothalamus

LPS injection increased hypothalamic expression of TNF-α, IL-1β, and IL-6, ($U = 19.00$, $p < 0.001$; $U = 0.00$, $p < 0.001$; $U = 30.00$, $p < 0.001$; Fig. 2G, H, and I respectively). Timed feeding did not alter hypothalamic cytokine expression, but did interact with LPS ($X^2 = 38.86$, $p < 0.001$; $X^2 = 43.57$, $p < 0.001$; $X^2 = 35.15$, $p < 0.001$; respectively), such that ad lib-fed mice did

not significantly increase TNF-α or IL-6 in response to LPS (Dunn's, $p > 0.05$). Relative to ad lib-fed mice, night-fed and day-fed mice increased cytokine expression in the hypothalamus in response to LPS (Dunn's, $p < 0.05$). Baseline expression of TNF-α, IL-1β, and IL-6 was not altered by timed feeding in the hypothalami of saline treated mice ($p > 0.05$).

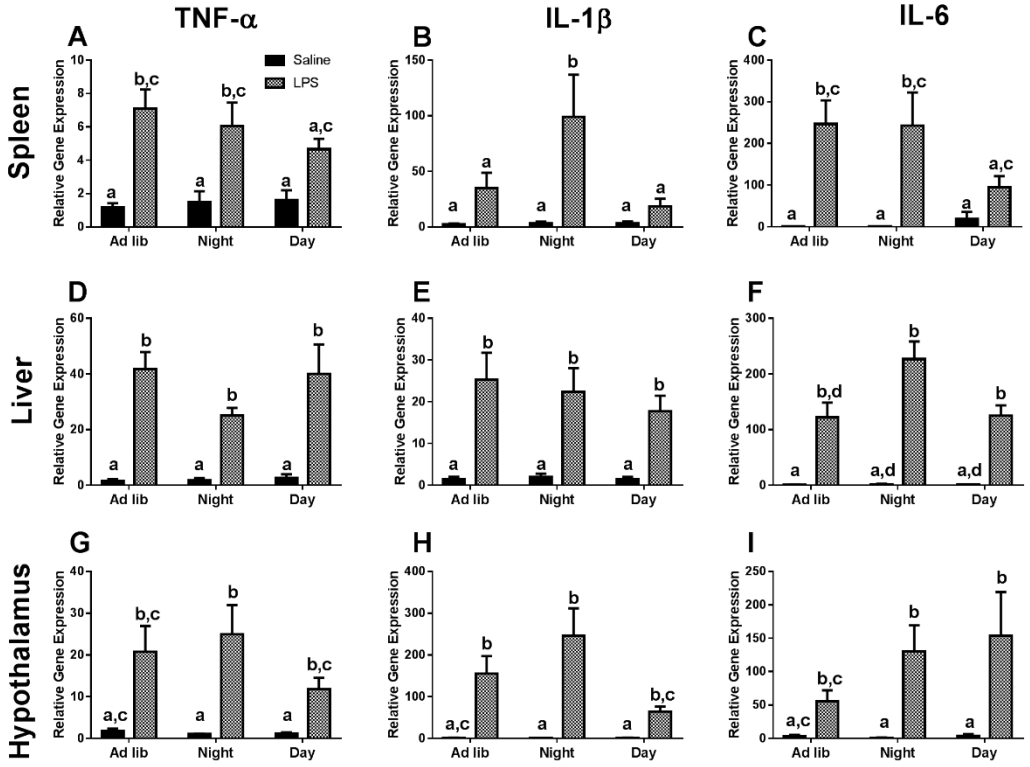

Figure 2

Bacteria Killing

LPS injection increased serum bactericidal capacity ($F_{1,29} = 9.22$, $p < 0.01$), specifically in the night-fed mice (Tukey's, $p < 0.05$).

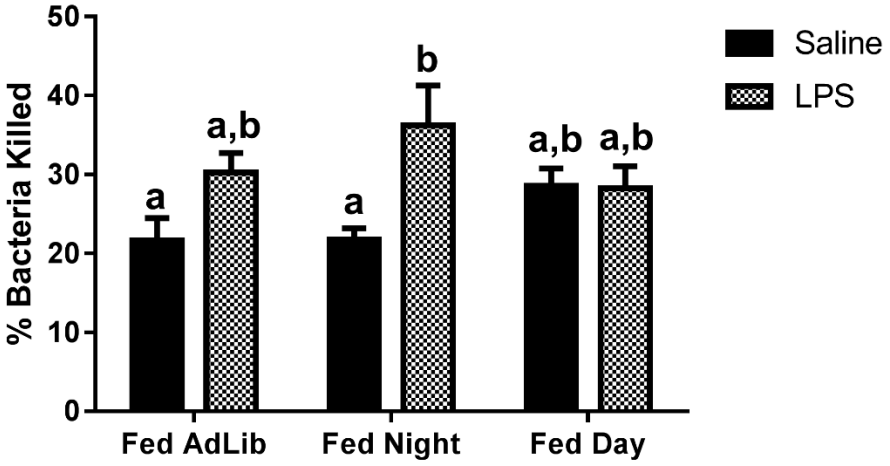

Figure 3

Discussion

Nocturnal mice typically ingest the majority of their food during the night. However, when mice were restricted to food only during the day, their cytokine response to LPS was impaired.. Changes in cytokine response were accompanied by a functional detriment in the bacteria killing capacity of these mice, in contrast with the significant increase in cytokine response and bacteria killing in other feeding groups after LPS injection relative to saline. These results occur without differences in body and adrenal mass suggesting that the results are independent of adiposity and activation of the HPA axis. They are also independent from light entrainment of circadian rhythms as all animals were exposed to the same light/dark cycle. These data suggest that limiting feeding to the inactive day phase in rodents has a detrimental effect on immune response, primarily in the spleen which is one of the primary immune response organs.

TNF-α is part of the acute immune response and is primarily secreted by macrophages. It is involved in several biological processes including apoptosis, lipid metabolism, and coagulation (22). Studies have also suggested a neuroprotective function of the cytokine TNF-α

(23). IL-1β is a proinflammatory cytokine released in the acute immune response (24). IL-6 is a cytokine important in the acute phase response. It is involved in inflammation and the maturation of B cells and found in sites of both acute and chronic inflammation (25). It is also involved in lipid breakdown (26). The expression of these cytokines in different tissues was assessed to compare the acute immune response between different feeding groups and between LPS and saline injected groups.

Body fat can influence immune responses. When there is less available energy due to lower adiposity, the immune system is suppressed in order to not consume energy needed for survival. Decreases in body fat impair humoral immunity in both the prairie vole and Siberian hamsters (27). There has been previously reported data of mice gaining weight when exposed to daytime feeding (28). However, this was in response to a high fat diet in addition to the time restricted feeding. In our study, there was no difference in overall body mass between the groups. The lack of body mass difference in our study suggests that there was no confounding variable of adiposity on the observed immune responses.

When animals are stressed, their hypothalamic-pituitary-adrenal (HPA) axis is activated and increased amounts of glucocorticoids are released. Glucocorticoids decrease immune response (29,30). There was no significant difference in adrenal mass between any of the feeding groups suggesting that difference in glucocorticoids were not a factor in the decreased immune response of day fed animals.

Spleen mass increases in response to immune challenges (31). Although there was an increase in spleen mass in the groups that received LPS as opposed to saline, there was no difference between the timed feeding groups. This suggests that the deficiency in immune response in day fed animals is not due to lack of cell expansion in the spleen, but possibly a

deficit in cytokine expression. Day fed animals decreased splenic expression of TNF-α, IL-1β, and IL-6 (Figure 2). However, night fed animals increased splenic expression IL-1β (Figure 2) and bacteria killing capacity (Figure 3) relative to *ad-lib* and day-fed mice suggesting that feeding only in the active phase is beneficial for inflammatory response.

The liver is also important in acute immune response to infection and inflammation. The complement system is the liver's central mechanism for immune response. This system is activated through an enzyme cascade to amplify the amount of complement proteins produced. These activated complement proteins then bind to the surface of the pathogen, signaling for engulfment of the pathogen by macrophages. Production of acute phase proteins in the liver is controlled primarily by inflammatory cytokines IL-1β, IL-6, and TNF-α (32). The liver also contributes to maintaining metabolic homeostasis and circadian regulation of metabolism (33). There was no difference in cytokine response between any of the feeding group. This suggests that the immunity response of the liver is not a driving factor in the bacteria killing differences mediated by time restricted feeding.

In response to infection, peripheral cytokines act on cytokine receptors in the hypothalamus to produce sickness behavior. This sickness response presents itself in animals exhibiting decreased locomotor, feeding, and social activities. At the three-hour time point, IL-1β was peaking in the hypothalamus. There appears to be a marginal decrease in expression of IL-1β in day-fed animals relative to other feeding groups. Day-fed animals did not exhibit LPS induced anorexia relative to their night-fed and day-fed counterparts suggesting that the sickness response mediated by the hypothalamus was dampened. There were no significant differences in expression of IL-6, or TNF-α among any feeding groups. These results suggest that there is a

deficit in the immune response of the hypothalamus when food intake is restricted to the inactive phase.

Night fed and *ad-libitum* fed mice showed a significant increase in bacteria killing in response to LPS in comparison to saline injected mice. Day fed mice, however, failed to show the same increased response to the LPS injection relative to the saline injection (Figure 3). This demonstrates a functional disruption in the immune response of day fed animals relative to night fed and *ad-lib* fed animals. These results coincided with cytokine expression in the spleen and hypothalamus and were independent of body and adrenal mass. This suggests that this decreased immune function is due to disruption of cytokine response and is independent from metabolic disruption and activation of the HPA axis.

In conclusion, our data provide evidence that time restricted feeding has an impact on immune response, specifically that day restricted feeding in mice has a detrimental effect. Animals that had their feeding restricted to their inactive period (day) did not display the same increase in bacteria killing as night fed and *ad-lib* fed animals in response to LPS compared to saline. This was reflected in a dampened expression of proinflammatory cytokines, primarily in the spleen of day fed animals relative to other feeding times following injection of LPS. In addition, day-fed mice did not exhibit a hypothalamus mediated anorexia response to LPS in comparison with other feeding groups. However, night-fed animals had an increased cytokine response and bacteria killing capacity relative to day-fed and *ad-lib* fed animals. Future studies should investigate whether timed feeding influences clock gene expression in immune organs and the downstream effects on immune function. These data expand on the influence of timed feeding, suggesting that in addition to metabolic disruption, it also contributes to a disruption in immune response.

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